Inhibition of Experimental Angiogenesis by the Somatostatin Analogue Octreotide Acetate (SMS 201-995)[1]

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ABSTRACT

The present study investigates the effect of the somatostatin analogue octreotide acetate (SMS 201-995) on experimental angiogenesis *in vitro* and *in vivo*. Octreotide reduced the proliferation of human HUV-EC-C endothelial cells (mean, −45.8% *versus* controls at 10^{-9} M; $P < 0.05$) as well as the density of the vascular network of the chick chorioallantoic membrane (mean, −35.7% *versus* controls at 50 μg; $P < 0.05$). Furthermore, octreotide significantly inhibited chick chorioallantoic membrane neovascularization by the human MCF-10A^{int-2} mammary cells secreting the angiogenic protein FGF-3. The proliferation of endothelial and smooth muscle cells from rat aorta explants on fibronectin was reduced by octreotide 10^{-8} M (mean, −32.6% *versus* controls; $P < 0.05$), and a similar effect was produced on cells sprouting from explants cultured in fibrin (mean, −52.9% *versus* controls; $P < 0.05$). Topical administration of octreotide 10 μg/day for 6 days inhibited rat cornea neovascularization induced by $AgNO_3/KNO_3$ (mean, −50.6% *versus* controls; $P < 0.05$). Octreotide 40 μg/day i.p was tested on angiogenesis in rat mesentery obtained by i.p. injections of compound 48/80, a mast cell degranulating agent, or conditioned medium from MCF-10A^{int-2} cells and was able to reduce the extent of neovascularization (mean, −45.6 and −64.1%, respectively, *versus* controls; $P < 0.05$). These data provide evidence that octreotide is an inhibitor of experimental angiogenesis *in vitro* and *in vivo*.

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INTRODUCTION

Somatostatin belongs to the large family of small regulatory peptides characterized by a wide spectrum of actions in the human body, including regulation of cell growth (1). After the landmark discovery of octreotide acetate (SMS 201-995), a synthetic somatostatin analogue with markedly increased biological half-life and GH[3]-suppressing activity (2), several studies have focused on the possible antiproliferative effect of this peptide based on the evidence that somatostatin receptors are detected in some human tumors (3).

Octreotide inhibits tumor growth *in vivo*, including ZR-75-1 breast cancer (4) and colon peritoneal carcinomatosis (5). Part of this effect might depend on the inhibitory activity on paracrine and/or autocrine growth factors, including EGF (6), bFGF (7), IGF-I (8), and on the increased production of the regulatory protein IGF binding protein I (9). Growth factor inhibition might be dependent on the presence of specific somatostatin receptors that mediate the dephosphorylation of substrates through the stimulation of a tyrosine phosphatase activity (10). Tumor-induced blood vessel growth is the result of the increased secretion of paracrine angiogenic peptides, including bFGF and IGF-I (11), and octreotide could be a candidate drug for angiogenesis suppression in view of its growth factor inhibition.

The antiangiogenic activity of octreotide has been demonstrated previously in the CAM (12, 13). In addition to this, octreotide treatment was able to reduce the progression of neovascularization associated with severe proliferative retinopathy in diabetic patients (14). The phenomenon of new vessel development encompasses a complex array of events, the regulatory mechanisms of which are incompletely understood. Therefore, the analysis of the antiangiogenic activity of a compound requires the use of several experimental models because substantial differences among them may be present, as well as response to putative antiangiogenic compounds. The aim of the present study was to test octreotide on *in vitro* and *in vivo* models of neovascularization to characterize its antiangiogenic effect.

MATERIALS AND METHODS

Materials. Media and supplements for cell culture, sodium alginate, the endothelial cell mitogen ECGF, human fibronectin, compound 48/80, bovine fibrinogen and thrombin, and all other chemicals not listed in this section were from Sigma Chemical Co. (St. Louis, MO). Plastics for cell culture were from Nunc (Ballestrup, Denmark). Octreotide acetate was a generous gift of Sandoz Pharma (Basel, Switzerland) and was

[3] The abbreviations used are: GH, growth hormone; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; IGF-I, insulin-like growth factor type I; ECGF, endothelial cell growth factor; CAM, chick chorioallantoic membrane; FGF-3, fibroblast growth factor-3; sst_2, somatostatin receptor subtype 2.

dissolved in sterile 0.9% NaCl as a vehicle. Female Wistar rats (body weight, 200 g) were from Nossan (Comerio, Italy) and were allowed unrestricted access to food and tap water; their care and handling were in accordance with the recommendations of the European Economic Community on animal experimentation.

Proliferation of HUV-EC-C Cells. The HUV-EC-C human endothelial cells (American Type Culture Collection, Rockville, MD) were cultured at 37°C and 5% CO_2 in 90% Ham's F12K, 10% fetal bovine serum, 30 μg/ml ECGF, 100 μg/ml heparin, and 4 mM L-glutamine. The effect of octreotide on HUV-EC-C cell proliferation was evaluated on 3×10^3 cells/well plated in 24-well plates. After 24 h, 10^{-10}–10^{-6} M octreotide or vehicle was added, and plates were incubated for 72 h. Cells were then harvested with trypsin/EDTA and counted by an hemocytometer. Results are expressed as number of cells in vehicle and octreotide-treated cultures and are the mean of three separate experiments ± SE. To investigate the influence of medium supplements on the effect of octreotide on HUV-EC-C cells, ECGF and heparin were either increased to 45 and 150 μg/ml or decreased to 15 and 50 μg/ml, respectively, and cell counting was performed.

Angiogenesis in the CAM. The technique of chick embryo treatment has been described previously (15). Briefly, embryos were cultured at 37°C in MEM with 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Agarose gel discs for sustained release of octreotide were obtained by mixing the somatostatin analogue with a sterile solution of low-melting point agarose 2.5%. Discs (5-mm diameter and 25-μl volume) contained 25–100 μg of octreotide or vehicle only for controls. Discs were placed on the CAM, and embryos were incubated for 48 h. The discs were gently removed to show the vascular architecture of the CAM underneath them, and a 20% fat emulsion (Lipofundin; Braun, Melsungen, Germany) was injected into the CAM to increase the contrast between blood vessels and surrounding tissues. Ten embryos/treatment were photographed for image analysis (see "Data Analysis").

Neovascularization of the CAM Induced by MCF-10A$^{int\text{-}2}$ Cells. Blood vessel growth was obtained by *int-2*-transfected MCF-10A cells (MCF-10A$^{int\text{-}2}$) secreting the angiogenic growth factor FGF-3, as reported earlier (16). Briefly, cells were mixed with 1.5% alginate sodium and 80 mM $CaCl_2$ to obtain a gel that is a suitable matrix for cells (17). Cell pellets (100 μl) were placed on the CAM of embryos obtained as described above. Octreotide (2 μg) was dissolved in 5 μl of MEM and injected every other day into the pellets by a syringe fitted with a 33-gauge needle, starting on the first day of implant; control pellets received MEM only. After 10 days, pellets were removed to allow a complete view of the neovascularization; 10 CAMs/treatment were injected with Lipofundin and photographed for image analysis (see "Data Analysis"). To document the viability of cells exposed to octreotide, the alginate matrix of the pellet was dissolved to recover MCF-10A$^{int\text{-}2}$ cells (17), and trypan blue (0.2%) dye exclusion technique was performed for viable cell counting.

Proliferation of Vascular Cells from Rat Aorta on Fibronectin. A method published previously has been adapted to the present study (18). Briefly, the thoracic aorta was excised from rats sacrificed with 1 g/kg i.p. urethane, and the fibroadipose tissue around the vessel was removed. One-mm-long rings of aorta were cut and placed in each well of a 96-well plate containing 90% DMEM, 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Plates were precoated with 0.3 μg/well of human fibronectin to improve cell proliferation. Octreotide (10^{-10}–10^{-6} M) was added after 5 days of culture and continued for 48 h; control explants received vehicle only. Cell proliferation was evaluated by the tetrazolium derivative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Cell Titer 96; Promega Corp., Madison, WI). Results are expressed as the percentage reduction of cell growth by octreotide relative to control cultures and are the mean of three experiments ± SE. To assess the relative proportion of endothelial *versus* smooth muscle cells, explants were analyzed by immunohistochemistry with anti-factor VIII and anti-actin (19).

Proliferation of Vascular Cells from Rat Aorta in Fibrin. This model has been described previously in detail (20). Briefly, 380 μl of fibrinogen (3 mg/ml) and 20 μl of thrombin (50 NIH units/ml) were added to each well of a 24-well plate. Rings of aorta were obtained as reported above and placed on the fibrin gel with the margin of section facing up. Additional clotting fibrinogen (0.4 ml) was added, followed by 500 μl of DMEM/Ham's F12 with 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. ε-Aminocaproic acid was included at 300 μg/ml for the first 3 days and at 50 μg/ml for the remainder of the study to inhibit the fibrinolytic activity of factors released by the aorta and growing vascular cells. The amount of ε-aminocaproic acid is required to be higher because the fibrinolysis is maximal during the first 3 days; then its concentration can be lowered without affecting the integrity of the fibrin clot. Octreotide was added at 10^{-10}–10^{-6} M after plating, and media were changed every other day; control wells received vehicle. Ten days later, 10 rings of aorta/treatment were photographed for image analysis (see "Data Analysis"). The explants were analyzed by immunohistochemistry with anti-factor VIII and anti-actin (19).

Neovascularization of Rat Cornea Induced by Chemical Injury. Growth of blood vessels was obtained as reported (21). Briefly, the cornea of anesthesized animals was cauterized by $AgNO_3$:KNO_3 (1:1, w/w) for 30 s at 2 mm from the corneoscleral limbus. Rats were immediately treated four times daily for 6 days with 2.5 μg/eye of octreotide in vehicle (0.9% NaCl, 0.1% cetylpyridinium HCl, and 2.5% carboxymethylcellulose) or vehicle alone. After the end of treatment, rats were sacrificed with 1 g/kg urethane i.p. and perfused through the thoracic aorta with Ringer's lactate to remove blood. Then Ringer's lactate containing 6% gelatin and 10% India ink was injected, and the eyes were frozen with dichlorodifluoromethane to solidify gelatin within the vessels. Eyes were removed and fixed in 4% phosphate-buffered formaldehyde (pH 7.4) for 24 h; then the cornea and 1-mm rim of scleral tissue were cut, and a total of 20 corneas/treatment were photographed for image analysis (see "Data Analysis"). A marked increase in bFGF immunoreactivity around the lesion is detected within 24 h after chemical damage of the cornea and before the growth of blood vessels; bFGF immunostaining slowly decreases during the following 2 days as the vascular network organizes (22).

Neovascularization of Rat Mesentery Induced by Compound 48/80. The i.p. injection of the mast cell-degranulating agent compound 48/80, a condensation product of *N*-methyl-*p*-methoxy-phenethylamine with formaldehyde, induces a marked angiogenic reaction in the rat mesentery (23). Compound 48/80 was given twice daily to animals starting at 1 mg/kg and increasing by 1 mg/kg to reach 5 mg/kg on the fifth day. Octreotide 20 μg twice daily was injected i.p. for 5 days, starting from the beginning of compound 48/80 challenge; control rats received vehicle only. Animals were then sacrificed with 1 g/kg urethane i.p., and the peritoneal cavity was exposed. Ten mesenteric windows, the triangular portions of mesentery delimited by the branches of mesenteric vessels and the small bowel, were randomly chosen and dissected from each rat after ligation of the vascular peduncle. The mesenteric windows from 10 rats/treatment were photographed for image analysis (see "Data Analysis").

Neovascularization of Rat Mesentery Induced by MCF-10A$^{int\text{-}2}$-conditioned Medium. The FGF-3 protein released by MCF-10A$^{int\text{-}2}$ cells induces angiogenesis in the rat mesentery, as described earlier (16). Briefly, conditioned medium was collected from MCF-10A$^{int\text{-}2}$ cells growing at 65–75% confluence for 5 days. Animals were injected i.p. with 1 ml of conditioned medium twice daily for 10 days and 20 μg i.p. octreotide twice daily or vehicle (0.9% NaCl). At the end of the study, 10 rats/treatment were sacrificed with 1 g/kg urethane i.p., and 10 mesenteric windows from each animal were dissected as reported above for image analysis (see "Data Analysis").

Data Analysis. Pictures of the CAM, rat cornea, and mesentery were obtained with a stereomacroscope (Zeiss, Oberkochen, Germany) at ×6.5-12; explants of aorta in fibrin were photographed with a phase-contrast microscope (Leica, Heerbrugg, Germany) at ×31.5-50. Each picture of the same magnification was analyzed with a Zeiss-Kontron KS-300-SW image analyzer (Zeiss, Oberkochen, Germany) as described (15, 21). Briefly, images were digitized and thresholded in the normalized gray level to distinguish vascular profiles from surrounding tissues. If needed, digitized images were optimized for analysis by erasing nonvascular structures and completing vascular profiles. The areas showing modification of the vascular network as a result of octreotide treatment were delineated, and their extension was calculated. Furthermore, the absorbances of images due to the presence of blood vessels were estimated by a gray scale analysis. The absolute values of surface area and absorbance were subjected to statistical analysis by ANOVA, followed by the Student-Newman-Keuls test, and then transformed in percentage of values relative to untreated samples. The level of significance was set at $P < 0.05$.

RESULTS

Octreotide Inhibited Proliferation of HUV-EC-C Cells. Octreotide 10^{-9} M produced a maximum 45.8% reduction of cell proliferation as compared to control cultures, from 9.7 to 4.4 × 10^3 cells/well ($P < 0.05$ *versus* controls; Fig. 1). The effect of increasing concentrations of octreotide on cell number was not linear, because the activity of the somatostain analogue was reduced at 10^{-10} M and below 10^{-8} M (Fig. 1). To assess the influence of medium supplements on the inhibition of HUV-EC-C cell growth, octreotide was tested against graded concentrations of ECGF and heparin in the culture medium. Overall, these changes did not significantly affect the growth-inhibitory activity of octreotide as compared to baseline conditions; in particular, the average reduction of cell proliferation obtained by combining 10^{-9} M octreotide with either ECGF/heparin 45/150 or 15/50 μg/ml was −41.2 and −48.5%, respectively, as compared to controls.

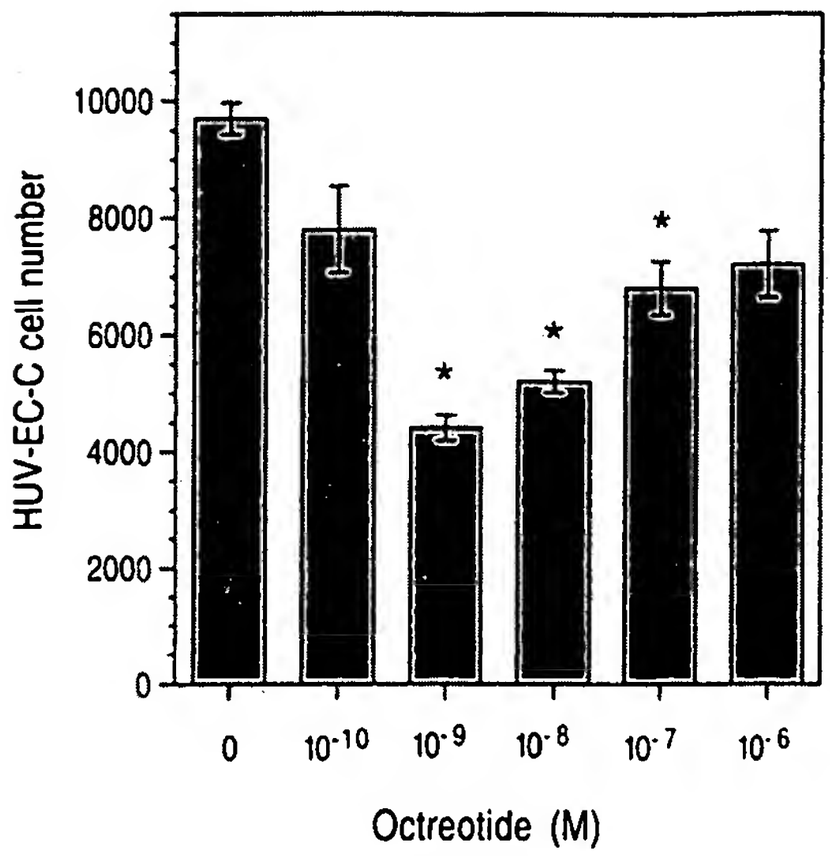


Fig. 1 Inhibition of HUV-EC-C endothelial cell proliferation by treatment with octreotide for 72 h. Data are shown as the number of cells in culture wells at the end of treatment and are the means of three separate experiments; *bars*, SE. *, $P < 0.05$ compared to vehicle-treated cells.

Octreotide Inhibited Angiogenesis in the CAM. Blood vessels formed a dense and spatially oriented, leaf-like branching network composed by vascular structures of progressively smaller diameter as they extend to the periphery of the CAM (Fig. 2). Treatment with octreotide started on the third day after embryo explant, when the CAM entered its phase of rapid growth. Octreotide (50 μg) produced an area of 1.1 ± 0.18 cm^2 with reduced density of blood vessel (−35.7 ± 6.9% *versus* controls; $P < 0.05$; Fig. 2). The total surface area of the CAM was 24.8 ± 5.9 cm^2 and was not significantly different between controls and octreotide-treated embryos. Octreotide inhibited the development of the tertiary and quaternary branches of vessels; the primary, structurally stabilized vessels were not affected, however. Likewise, 100 μg octreotide reduced the absorbance of blood vessels (−42.1 ± 5.4% *versus* controls; $P < 0.05$) over a surface of 5.7 ± 0.7% of the CAM, whereas 25 μg octreotide resulted in a modest inhibition of angiogenesis (−14.8 ± 2.5% *versus* controls) over an area of 1.7 ± 0.2% of the total CAM.

Octreotide Inhibited Neovascularization of the CAM Induced by MCF-10A$^{int\text{-}2}$ Cells. The alginate gel matrix allowed the entrapment of a large number of cells in a small volume while allowing the release of the angiogenic factor FGF-3, the translation product of the *int-2* oncogene secreted by MCF-10A$^{int\text{-}2}$ cells. Neovascularization was obtained within 8–12 days after implant of cell pellets and consisted of a large

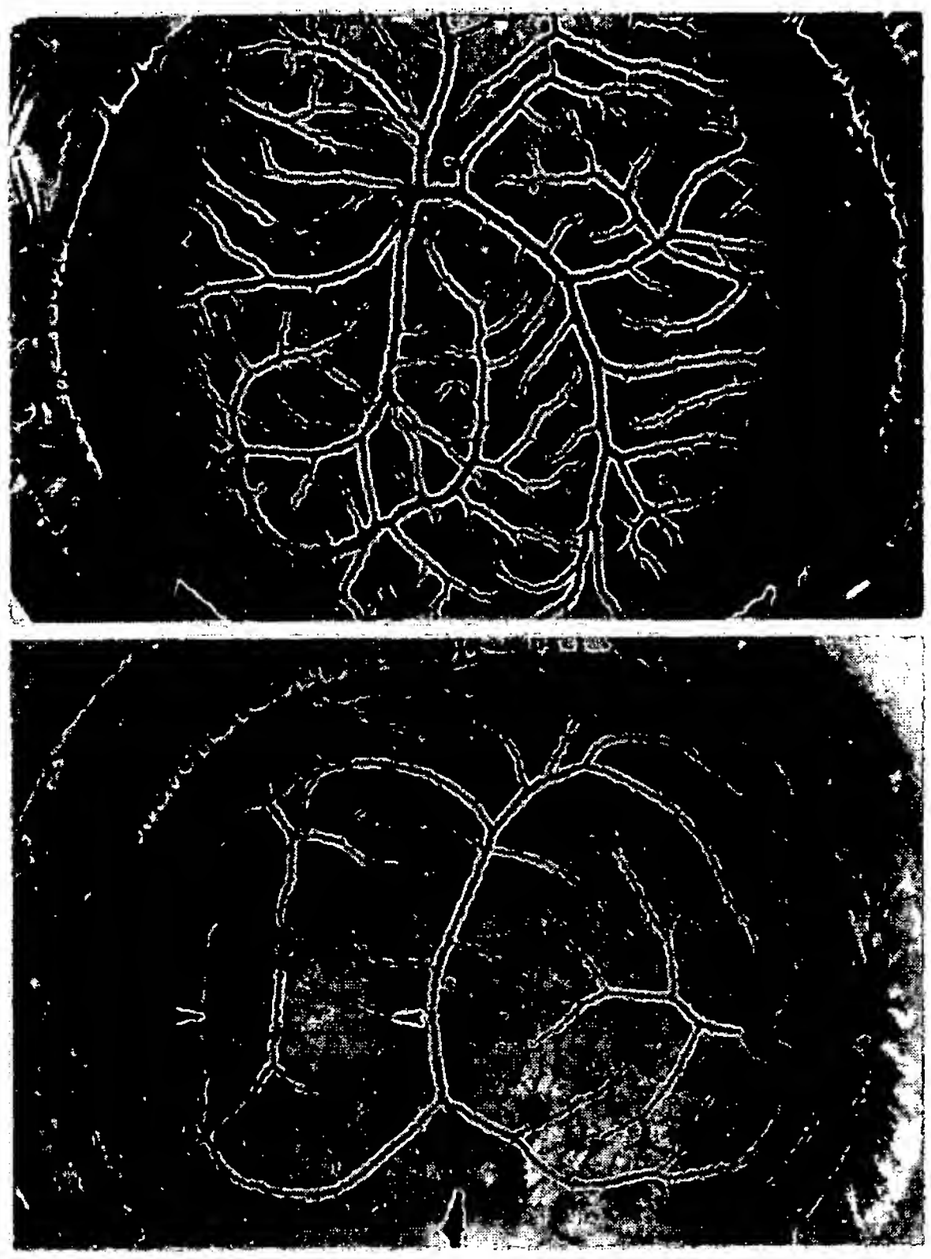


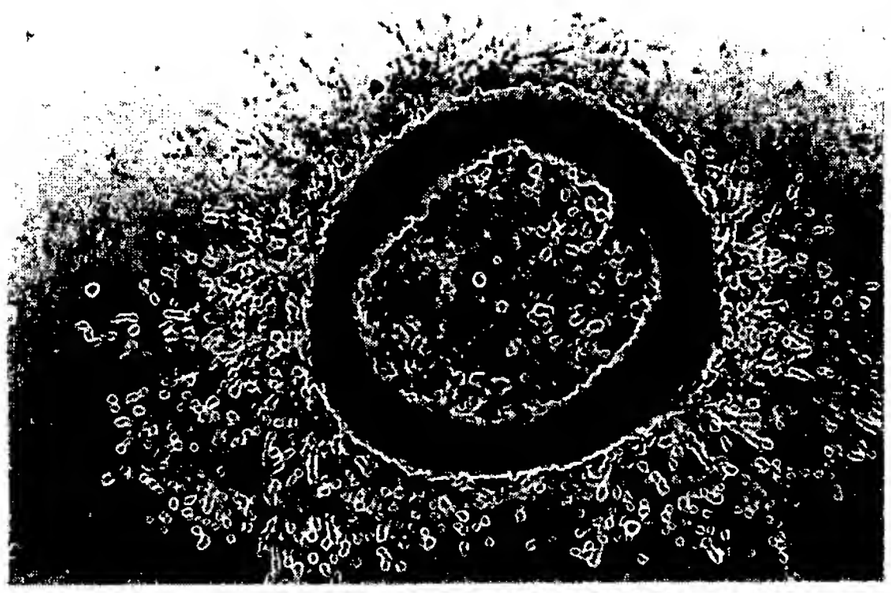
Fig. 2 Angiogenesis in the CAM. The leaf-like branching pattern of blood vessels of the control CAM is characterized by numerous capillaries originating from primary and secondary vessels (*top*). *Bottom*, inhibition of angiogenesis in the CAM by 50 μg octreotide for 48 h. The branching pattern of blood vessels is inhibited, and the faint boundaries and capillaries of the CAM (*arrowheads*) are markedly reduced. ×10.

Fig. 3 Angiogenesis induced in the CAM by application of an alginate disc containing MCF-10A$^{int\text{-}2}$ cells. Short capillaries originating from secondary vessels grew toward the cell pellet (*arrowheads*) in untreated CAM (*top*). *Bottom*, inhibitory effect of 2 μg octreotide injected every other day for 10 days in the disc containing MCF-10A$^{int\text{-}2}$ cells. The treatment produced a marked decrease in angiogenesis (*arrowheads*). ×10.

number of short, tiny capillaries originating from the secondary branches of blood vessels of the CAM and directing toward MCF-10A$^{int\text{-}2}$ cells (Fig. 3). Injections of 2 μg octreotide into the pellets produced an area of marked inhibition of tertiary and quaternary branches of CAM vessels (Fig. 3). The hypovascular area produced by octreotide was 0.62 ± 0.08 cm^2, whereas the absorbance of blood vessels in this area was reduced (−58.7 ± 6.5%; $P < 0.05$ *versus* controls). The viability of MCF-10A$^{int\text{-}2}$ cells in alginate pellets, as assessed with the trypan blue dye exclusion technique, resulted in the conclusion that the cells were not significantly affected (10% cells in control and 14% in octreotide-treated pellets were stained by the dye; $P > 0.05$).

Octreotide Inhibited Proliferation of Vascular Cells from Rat Aorta on Fibronectin. A mixed population of smooth muscle cells (>87% of the total population) and endothelial cells originated from the margins of section of the explants and formed a monostrate on the surface of the culture wells (Fig. 4). Their attachment and growth was greatly improved by coating wells with human fibronectin. Cell proliferation entered the log phase of growth after an average of 4 days after the explant and reached the plateau in 8 days. Treatment with octreotide produced a significant inhibition of cell proliferation, with a maximum effect of −32.6 ± 5.8% *versus* control values ($P < 0.05$), at 10^{-8} M (Fig. 4). The profile of drug-induced inhibition of cell growth was not linear; the reduction of vascular cell proliferation obtained with 10^{-9} and 10^{-7} M octreotide was −24.2 ± 4.5 and −21.2 ± 2.1%, respectively ($P < 0.05$ *versus* controls in both cases), whereas no significant effects were noted at 10^{-10} and 10^{-6} M.

Octreotide Inhibited Proliferation of Vascular Cells from Rat Aorta in Fibrin. A large number of cells originated from the margins of section of rat aorta as well as from the aortic branches remaining after stripping off the paraaortic fibroadipose tissue. Vascular structures penetrated the fibrin gel and formed a rich network around the rings of aorta (Fig. 5). Cell population was composed of smooth muscle cells (>85% of the total), and the remaining portions were endothelial cells. Microvessel growth fully developed 1 week later and then reached a plateau after 14 days. The area of microvessels in control explants at the tenth day of culture was 1.7 ± 0.3 mm^2. Cultures treated with 10^{-8} M octreotide produced a thin crown of microvessel sprouts around the rings of aorta and rapidly reached the plateau phase (Fig. 5). The vascular area (0.8 ± 0.1 mm^2, −52.9 ± 7.2% *versus* controls) as well as the absorbance (−33.2 ± 4.7% *versus* controls) were significantly reduced



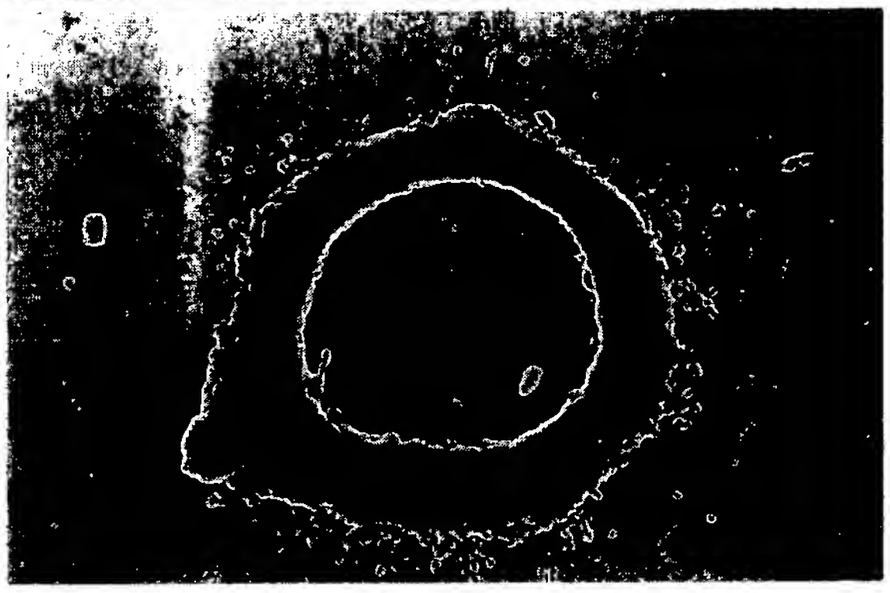


Fig. 4 Growth of vascular cells from segments of rat aorta on fibronectin. A monostrate of endothelial and smooth muscle cells originated from an untreated explant (*top*). *Bottom*, inhibition of vascular cell growth by 10^{-8} M octreotide for 48 h. The treatment produced a sparse outgrowth of cells. ×40.

Fig. 5 Growth of microvessels from segments of aorta in fibrin. A dense microvascular network composed of branching channels proliferating into the fibrin clot (*arrowheads*) originated from an untreated explant (*top*). *Bottom*, inhibition of microvessel growth by 10^{-8} M octreotide for 10 days. The treatment produced a sparse outgrowth of cellular sprouts (*arrowheads*) penetrating into the fibrin meshwork. ×50.

($P < 0.05$). Octreotide (10^{-9} and 10^{-7} M) inhibited vascular cell growth (-18.6 ± 4.5 and $-27.2 \pm 3.8\%$, respectively; $P < 0.05$ *versus* controls), whereas no significant changes were observed at 10^{-10} and 10^{-6} M.

Octreotide Inhibited Neovascularization of Rat Cornea Induced by Chemical Injury. After application of $AgNO_3$/KNO_3, small blood vessels originated from the limbar vascular arcades and invaded the adjacent cornea of control animals during the second and third day. During the following 4 days, a dense capillary network spread within the cornea and directed toward the lesion (Fig. 6); the area of the cornea containing blood vessels was 1.6 ± 0.3 mm^2. Topical treatment with octreotide 10 μg/day/eye for 6 days significantly inhibited angiogenesis; vascular sprouts were reduced in number, and their spread was limited to the cornea adjacent to the corneoscleral limbus (Fig. 6). The area of blood vessels was significantly reduced (0.79 ± 0.1 mm^2; $-50.6 \pm 4.7\%$ *versus* controls; $P < 0.05$), whereas the absorbance showed a modest change ($-16.7 \pm 4.3\%$ *versus* controls). In this model, a marked increase in bFGF immunoreactivity is detected in mesenchymal cells and surrounding stroma around the lesion before the development of blood vessels. This finding suggests that corneal damage indeed produces an increase in the angiogenic factor bFGF that might be responsible for promoting neovascularization (22).

Octreotide Inhibited Neovascularization of Rat Mesentery Induced by Compound 48/80. The normal mesentery is composed of a single layer of mesothelial cells possessing a basal membrane sandwiching an extracellular matrix rich in collagen bundles and containing fibroblasts and mast cells as well as microscopic vascular structures. The injection of compound 48/80 i.p. resulted in the formation of large vessels and hemorrhages within a thickened mesentery (Fig. 7). The vascular growth appeared to be significantly less intense if octreotide was administered together with compound 48/80 (Fig. 7). Image analysis of tissue samples after treatment with compound 48/80 demonstrated that the blood vessel area was 12.5 ± 2.9 mm^2/mesenteric window, whereas it was significantly reduced by 40 μg/day octreotide for 5 days (6.8 ± 0.5 mm^2/mesenteric window; $P < 0.05$ *versus* compound 48/80). Likewise, the absorbance of mesenteric windows in rats given compound 48/80 was reduced by treatment with octreotide ($-35.9 \pm 2.3\%$; $P < 0.05$ *versus* compound 48/80).

Octreotide Inhibited Neovascularization of Rat Mesentery Induced by MCF-10A$^{int\text{-}2}$-conditioned Medium. The i.p. injection of culture medium from MCF-10A$^{int\text{-}2}$ cells secreting the angiogenic protein FGF-3 elicited the growth of long, tortuous vessels with anastomoses and branching capillar-

Fig. 6 Angiogenesis in the $AgNO_3/KNO_3$-cauterized cornea. The pericorneal vessels are dilated, and an extensive network of packed capillaries penetrates into the cornea and forms loops toward the site of cautery (*arrowheads*) in control rats (*top*). *Bottom*, inhibition of angiogenesis by 10 μg octreotide/eye daily for 6 days. Few vessel loops penetrate into the cornea (*arrowheads*). ×25.

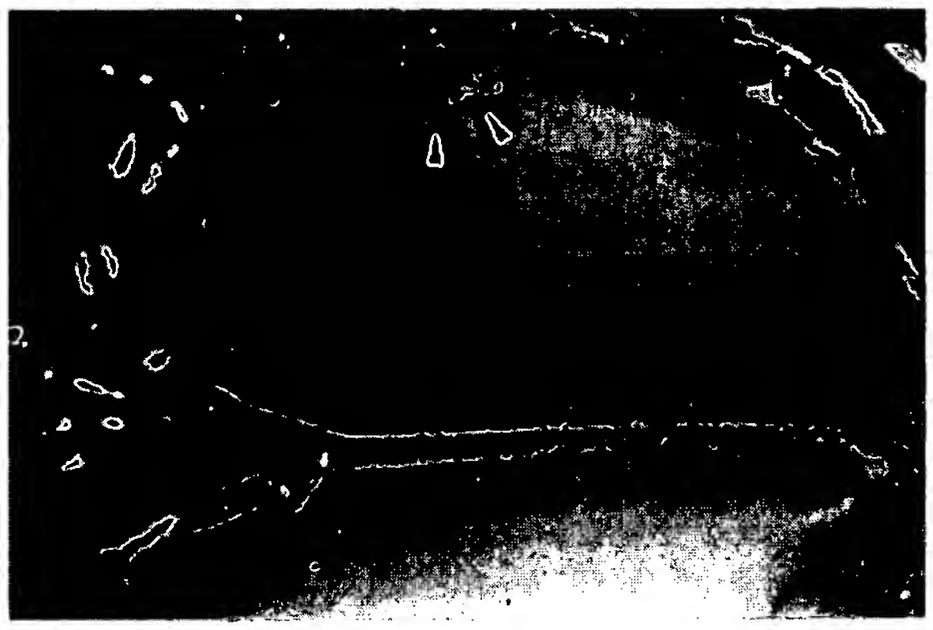


Fig. 7 Angiogenesis in the rat mesentery induced by compound 48/80. A capillary network (*arrowheads*) developed throughout the stromal layer of the mesentery in control animals (*top*). *Bottom*, inhibition of compound 48/80-induced angiogenesis by 40 μg i.p. octreotide daily for 5 days. The vascular growth reaction (*arrowheads*) is reduced as compared to compound 48/80 alone. ×12.

ies within the stromal layer of the mesentery (Fig. 8). This model allowed the characterization of the ability of octreotide to inhibit the effects of the paracrine angiogenic factor. The vascular area of control rats was 5.3 ± 0.2 mm^2/mesenteric window. Octreotide (40 μg/day i.p.) for 10 days significantly reduced blood vessels growth (Fig. 8); the vascular area was 1.9 ± 0.2 mm^2/mesenteric window, and the absorbance of vascularized tissue was reduced by −15.9 ± 2.3% ($P < 0.05$ *versus* animals receiving conditioned medium only).

DISCUSSION

The understanding of angiogenesis has expanded greatly over the past two decades due to the development of *in vivo* and *in vitro* models to study this process. A variety of cytokines and growth factors has been shown to induce new blood vessel formation *in vivo*, and the improved knowledge of the mechanisms which regulate angiogenesis has created important opportunities to develop new therapies for the treatment of cancer.

It is now well recognized that the vascular supply is an essential requirement for the growth of malignancies and the development of metastasis (24). Before vascularization, the growth rate of tumors is linear and limited, and rapid growth starts only after the development of an appropriate vascular supply (25). A distinctive feature of tumor cells is their capacity of eliciting continued growth of new blood vessels from the host; this characteristic is an important factor of neoplastic transformation (26). Angiogenesis is under a complex and multifactorial regulation; *in vivo*, quiescent endothelial and smooth muscle cells are stimulated to proliferate by angiogenic factors secreted by tumor cells or released from the extracellular matrix (26), and a partial list includes members of the FGF family, EGF/transforming growth factor α, platelet-derived growth factor, IGF-I, transforming growth factor β, and vascular endothelial growth factor, one of the most active angiogenic peptides (25, 26).

A treatment that would prevent vascular cells from responding to angiogenic stimuli appears to be a rational approach; indeed, almost all angiogenesis inhibitors characterized thus far belong to this class of anti-endothelial agents (15, 26, 27). Another reason that makes this approach the most feasible is that the tumor vasculature exhibits a phenotype that is markedly different from the quiescent endothelium because it is exposed to a multitude of angiogenic factors released by the malignant tissue (26). Nonetheless, the endothelium in tumor stroma is composed of a nontransformed cell population that

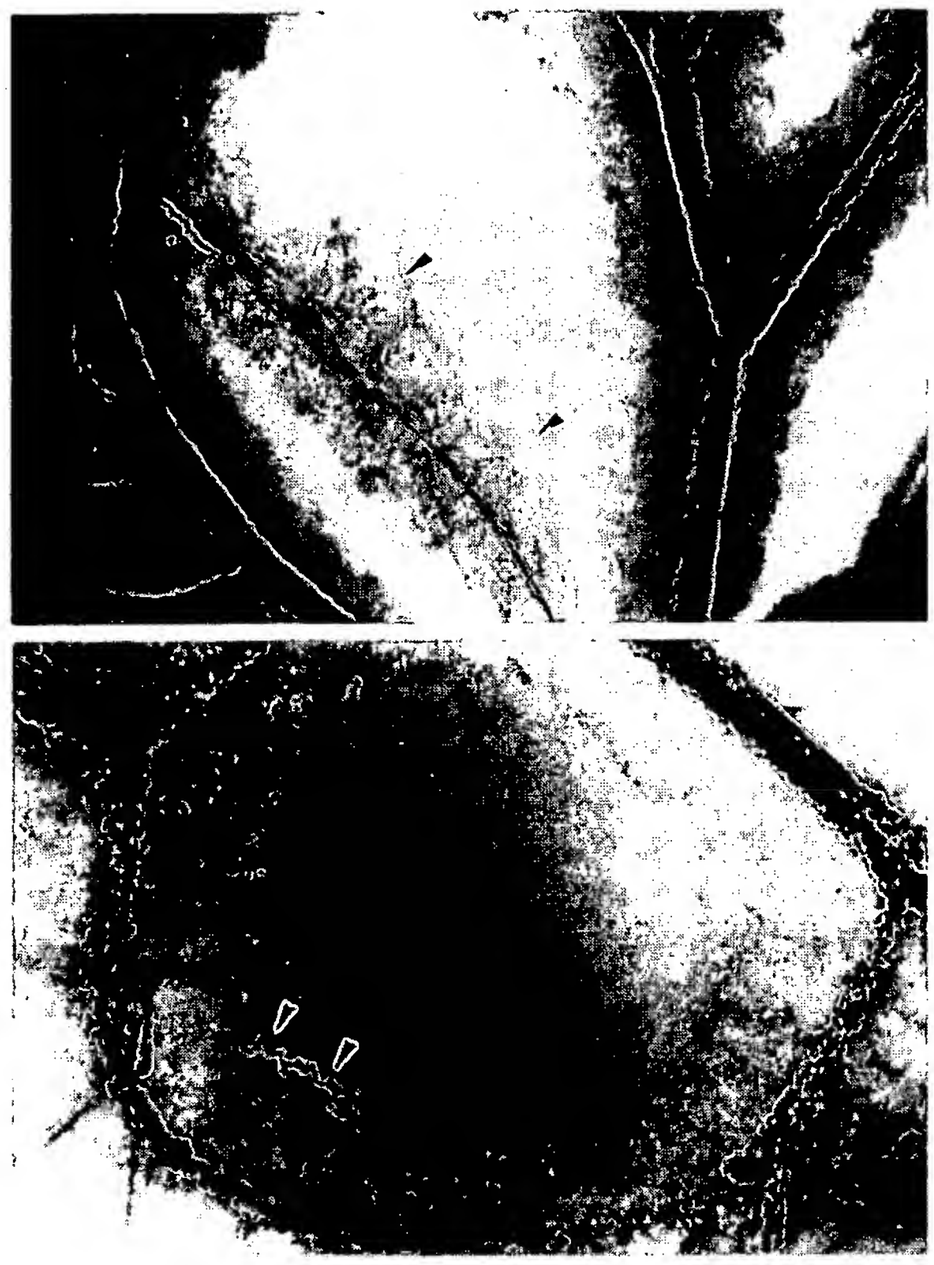


Fig. 8 Angiogenesis in the rat mesentery by i.p. injections of conditioned medium from MCF-10A^{ini-2} cells. The treatment induced the growth of a capillary network (*arrowheads*) developing throughout the stromal layer of the mesentery of control animals (*top*). *Bottom*, inhibition of angiogenesis by 40 μg i.p. octreotide daily for 10 days. Neoformed blood vessels (*arrowheads*) are reduced as compared to controls. ×12.

could be easier to target, compared to the genotypically and phenotypically instable malignant cells.

The data obtained in the present study indicate that octreotide was able to inhibit the proliferation of vascular cells *in vitro*, including the HUV-EC-C cell line as well as the endothelial and smooth muscle cells originating from explants of rat aorta. The relationship between octreotide concentrations and growth-inhibitory activity on vascular cells was not linear, however, a finding that might represent a limitation on its use *in vivo*. A similar behavior has been observed previously in colon and pancreas cancer cells *in vitro* (6, 7). However, the inhibitory activity of octreotide on the *in vivo* models of angiogenesis of the present study demonstrates that the somatostatin analogue did not suffer such a limitation. Indeed, octreotide was able to reduce the growth of endothelial and smooth muscle cells as well, providing evidence that the somatostatin analogue was effective on both cell types that represent the normal population of blood vessels.

The findings of this study suggest that the inhibition of the activity of a paracrine angiogenic factor belonging to the FGF family represents, at least in part, the cellular mechanism by which octreotide exerts its effects on blood vessel growth *in vivo*. This hypothesis is based on the results obtained in the neovascularization of the CAM and rat mesentery produced by the growth factor FGF-3 released by MCF-10A^{ini-2} cells. Furthermore, the marked reduction exerted by octreotide on the vasculogenic effect of compound 48/80 in the rat mesentery and of $AgNO_3/KNO_3$ in the rat cornea may indeed depend on the inhibition of the cellular effects of a variety of angiogenesis factors. Tumor models of neovascularization were not included in the present study because of the possible inhibitory effect of octreotide on tumor growth. In this respect, there is as yet no reliable method to distinguish the effect of a drug on blood vessel supply from that on cancer cell proliferation. Nonetheless, the present findings provide evidence that octreotide reduced the blood vessel growth driven not only by FGF-3 but also by multiple factors released under various experimental conditions *in vivo*. This result is important because a large number of peptides are found to promote blood vessel growth *in vitro* and *in vivo*, and the FGF family represents only a part of these factors (24)

The postreceptor signal transduction mechanisms involved in the antiangiogenic effect of octreotide were investigated in the CAM by Patel *et al.* (28). The ability of octreotide to reduce vessel growth was diminished by the inhibition of G-protein activity and increase of Ca^{2+} and cyclic AMP; on the contrary, octreotide was not affected by modulation of the activity of protein kinase C or tyrosine phosphatase (28). Adenylyl cyclase activity and Ca^{2+} mobilization are controlled by all somatostatin receptor subtypes (29); hence, the predominant antiangiogenic effect displayed by sst_2-preferring somatostatin analogues, including octreotide, might be dependent on the prevalence of the sst_2 receptor on vascular cells, a finding that has been demonstrated previously in tumor stroma (1).

The only clinical evidence available thus far on the possible antiangiogenic effect of octreotide has been provided by the study of Mallet *et al.* (14) in subjects suffering from proliferative diabetic retinopathy resistant to photocoagulation. Patients were treated with octreotide for a mean duration of therapy of 15 months, and GH monitoring demonstrated a progressive decline of hormone levels over time, up to a 57% reduction compared to baseline. The proliferative retinopathy underwent stabilization in two patients, and visual acuity improved in all of them. The findings of the present study provide additional experimental support to the clinical evidence of angiogenesis inhibition by octreotide and strengthen the link between the sst_2-preferring ligands and the control of neovascularization. It is worthwhile to mention that medical hypophysectomy obtained by treatment with sst_2-preferring somatostatin analogues is able to reduce the secretion of IGF through the suppressive effect on GH (1). The clinical observation by Mallet *et al.* (14) has received substantial support by the evidence that IGF-I is a growth factor for human retinal endothelial cells in culture and that the mitogenic effects of IGF-I and bFGF *in vitro* are inhibited by octreotide (30). Therefore, the suppression of neovascularization by octreotide *in vivo* could be dependent in part on a direct inhibition of IGF-I activity on endothelial cells or GH-mediated production of IGF-I, or both.

The therapeutic activity of octreotide on tumor growth *in vivo* has been suggested to depend at least in part on its effect on blood vessel development. This assumption was based on the finding that octreotide inhibited the angiogenesis in the CAM (12, 13), a model that suffers from inherent limitations. The

present study provides substantial evidence that octreotide exerts a significant antiangiogenic effect *in vitro* and *in vivo* that might contribute to its antiproliferative activity *in vivo*. It should be pointed out, however, that in addition to the inhibition of blood vessel supply, a significant portion of the antiproliferative effect of octreotide is dependent on its ability to affect tumor cells via receptor-mediated mechanisms acting on the mitogenic effects of growth factors such as bFGF, EGF, IGF-I, and gastrin (6–8, 31). In addition to this, octreotide enhanced the antitumor effects of tamoxifen or ovariectomy in mammary cancer induced by the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene in rats; the effect of octreotide was most evident in reducing the rate of tumor regrowth after ovariectomy and increasing tumor response rate when combined to tamoxifen (32).

In conclusion, the findings obtained in the present study indicate that the somatostatin analogue octreotide is able to inhibit blood vessel growth under a variety of experimental conditions and provide additional evidence that the antiproliferative effect of octreotide on tumor models *in vivo* may be mediated, at least in part, by its suppressive effect on blood vessel supply.

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